THE GLYOXYLIC ACID REACTION FOR TRYPTOPHAN, INDOL, AND SKATOL.

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(Received for publication, August 16, 1906.)

The reaction for the detection of proteins originally described by Adamkiewicz \(^1\) consisted in the addition of strong sulphuric acid to the acetic acid solution of the substance under investigation. In the presence of proteins a reddish violet ring is produced at the junction of the fluids which spreads throughout the solution on standing or on gentle shaking. This test was subsequently investigated by Hopkins and Cole \(^2\) who found that proteins did not uniformly yield a positive reaction and that the differences were attributable to the acetic acid employed in the test. They found that pure acetic acid did not give the reaction and so they were led to the belief that an essential condition for the success of the Adamkiewicz reaction was the presence of some substance commonly found as an impurity in acetic acid. As is well known, they eventually concluded that this impurity was glyoxylic acid, for they found that a dilute solution of glyoxylic acid gave the reaction in a typical fashion and that the presence of acetic acid was unnecessary.

By the subsequent isolation of tryptophan from the products of protein hydrolysis, Hopkins and Cole \(^3\) were able to show that the Adamkiewicz reaction was due to the presence of tryptophan groupings in the protein molecule, for not only did tryptophan itself show the same reaction with glyoxylic acid, but positive results were obtained with all proteins containing the tryptophan grouping but not with gelatin, which yields no tryptophan.

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\(^3\) Journ. of Physiol., xxvii, p. 418, 1900; xxix, p. 451, 1902.
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Recently, however, a paper has appeared by Rosenheim \(^1\) in which the correctness of some of the results of Hopkins and Cole is questioned. Rosenheim shows that a typical Adamkiewicz reaction may be obtained by the addition of commercial sulphuric acid to a protein or tryptophan solution to which a minimal quantity of formaldehyde has been added. He finds, however, that pure sulphuric acid and pure formaldehyde do not yield the reaction as the presence of some oxidizing agent is necessary. The oxidizing substance may be conveniently introduced into pure sulphuric acid by the addition of traces of ferric chloride, hydrogen peroxide, or potassium persulphate, etc. Excess of formaldehyde must be avoided as under these conditions the reaction is interfered with.\(^2\)

Rosenheim considers that the Adamkiewicz reaction as previously obtained with commercial acetic acid or with glyoxylic acid was really due to the presence of formaldehyde. He further states that he has had three samples of "pure" glyoxylic acid which gave no reaction when tested with proteins and pure sulphuric acid but it must be noted that no information is given as to the method of preparation of any of these specimens, nor is any evidence offered of their purity. A sample of glyoxylic acid prepared by the reduction of oxalic acid gave the reaction, but this Rosenheim ascribes to the presence of persulphuric acid, or hydrogen peroxide.

As the writer\(^3\) made use of the reaction with skatol and with tryptophan, in conjunction with other tests for the detection of glyoxylic acid, it was of importance to inquire into the correctness of Rosenheim's conclusions. Although Rosenheim's results with regard to the reactions between formaldehyde, proteins or tryptophan, and sulphuric acid are confirmed, it has not been possible to obtain any evidence in support of his statement that glyoxylic acid does not yield the reaction. Experience with this reaction completely confirms the original statements of Hopkins and Cole. Glyoxylic acid or its salts have been prepared in many different ways and in all cases the products have given an

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2 The Hehner test for the detection of formaldehyde in milk and its many modifications are merely applications of the tryptophan-formaldehyde reaction. Cf. Acree, This Journal, ii, p. 145, 1906.
3 This Journal, i, p. 271, 1906.
extremely delicate reaction with tryptophan (or protein) and strong sulphuric acid. The following methods of preparation have been employed:

I. The oxidation of alcohol with nitric acid.

II. The reduction of oxalic acid with sodium amalgam.

III. The electrolytic reduction of oxalic acid.

IV. The action of water upon dibromacetic acid.

V. The action of water upon dichloracetic acid.

VI. The action of silver oxide upon dichloracetic acid.

VII. The action of water upon silver dichloracetate.

The first two methods were used by Hopkins and Cole in the preparation of their acid but Rosenheim considers that "the methods employed by Hopkins and Cole for the preparation of glyoxylic acid do not absolutely exclude the presence of oxidizing agents." This objection can, however, hardly be applied to all the other methods of preparation. Using the purest glyoxylic acid (or its salts) that I have been able to obtain, I find that the reaction with tryptophan is as sensitive as Rosenheim's formaldehyde reaction and that colored products are also obtained with skatol and indol. Skatol yields a bright pink-red coloration which does not change on dilution with water, while indol yields a somewhat darker red color, and α-methylindol also yields colored products but the reaction in this case is much less sensitive. The relationship between these substances is seen from the following formulæ:

\[
\text{Tryptophan} \quad \text{NH} \quad \text{NH}_2 \quad \text{COOH}
\]

\[
\text{Skatol} \quad \text{NH} \quad \text{CH}_3
\]

\[
\text{α-Methylindol} \quad \text{NH} \quad \text{CH}_3
\]

\[
\text{Indol} \quad \text{NH}
\]

Brief reference to a few points in the preparation of glyoxylic acid by the different methods may be made.

The electrolytic reduction of oxalic acid was performed in sulphuric acid solution according to Traube's directions, using a divided cell and leaden electrodes.\textsuperscript{1} It is most important that rise in temperature inside the cathode chamber be avoided as otherwise glycollic acid forms the main product of reaction. At the end of the reduction, when no more oxalic acid was present, the liquid was diluted, exactly freed from sulphuric acid by barium hydroxide, and the free glyoxylic acid was then converted into the calcium salt which was purified by repeated crystallization from water. The product in every case gave a typical reaction with tryptophan, indol, and skatol in extremely dilute solution.

Dibromacetic acid was prepared by the direct bromination of pure acetic acid.\textsuperscript{2} The product was fractionated under ordinary pressure and the portion boiling at 230–235\degree C. was used. After simply boiling with water or better by heating in a sealed tube with water at 135\degree C.,\textsuperscript{3} the solution gave an intense reaction with tryptophan and strong sulphuric acid.

The action of silver oxide upon dichloracetic acid has been investigated by Beckurts and Otto.\textsuperscript{4} Two molecular proportions of pure dichloracetic acid (i.e. an excess) which had been prepared by the action of potassium cyanide upon chloral\textsuperscript{5} were boiled with one molecular proportion of freshly precipitated silver oxide, suspended in water. Decomposition takes place in accordance with the following equation:

\[
\text{CHCl}_2\text{COOH} + \text{Ag}_2\text{O} \rightarrow \text{C} = \text{COOH} + 2\text{AgCl}
\]

\[
\text{CHBr}_2\text{COOH} + \text{H}_2\text{O} \rightarrow \text{C} = \text{COOH} + 2\text{HBr}
\]

\textsuperscript{2} Perkin and Duppa, \textit{Ann. d. Chem.}, cx, p. 115, 1859.
\textsuperscript{3} Grimaux, \textit{Bull. de la soc. chim.} (Paris), xxvi, p. 483, 1876.
\textsuperscript{5} Chloral itself gives no reaction with tryptophan and strong sulphuric acid.
The silver chloride was filtered off and a trace of the filtrate was found to react in a typical manner with tryptophan and sulphuric acid. In order to separate the glyoxylic acid from the excess of dichloracetic acid, the acids were converted into their calcium salts by means of calcium carbonate. On concentration, well-formed crystals of calcium glyoxylate crystallized out and were separated from the more soluble calcium dichloracetate. The calcium salt was twice recrystallized from water and was then tested in the following way:

Reaction with Tryptophan. A solution of pure crystallized tryptophan was used, containing one-tenth of a milligram in one cubic centimeter. One cubic centimeter of this solution was added to one cubic centimeter of calcium glyoxylate solution and then two to two and one-half cubic centimeters of pure sulphuric acid were added. The color at the contact zone was first observed and then the liquids were caused to slowly mix by gentle shaking. In a second series of experiments, one cubic centimeter of a calcium glyoxylate solution containing one-tenth of a milligram was used and the amount of tryptophan was varied. The results were as follows:

<table>
<thead>
<tr>
<th>Calcium Glyoxylate (Grams)</th>
<th>Tryptophan (Grams)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>0.0001</td>
<td>Intense reaction.</td>
</tr>
<tr>
<td>0.0001</td>
<td>&quot;</td>
<td>Strong reaction.</td>
</tr>
<tr>
<td>0.000005</td>
<td>&quot;</td>
<td>Slight reaction.</td>
</tr>
<tr>
<td>0.000001</td>
<td>&quot;</td>
<td>Slight but positive reaction.</td>
</tr>
<tr>
<td>0.0001</td>
<td>0.00005</td>
<td>Strong reaction.</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.00001</td>
<td>Slight but positive.</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.000005</td>
<td>Just positive.</td>
</tr>
</tbody>
</table>

The results show that calcium glyoxylate prepared in the way described reacts in a typical manner with one-tenth of a milligram of tryptophan at a dilution of one part in two hundred thousand and conversely using one-tenth of a milligram of calcium glyoxylate, one part of tryptophan in two hundred thousand parts of water may be detected.

The same specimen of calcium glyoxylate was tested with indol and with skatol. The sensitiveness of the test with indol is approximately the same as that with tryptophan, but with
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skatol the reaction is still perceptible at a slightly greater dilution.

<table>
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<th>Calcium Glyoxylate.</th>
<th>Skatol.</th>
<th>Result.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grams.</td>
<td>Grams.</td>
<td></td>
</tr>
<tr>
<td>0.0001</td>
<td>0.001</td>
<td>Intense pink-red solution.</td>
</tr>
<tr>
<td>0.0001</td>
<td>&quot;</td>
<td>Strong reaction.</td>
</tr>
<tr>
<td>0.00005</td>
<td>&quot;</td>
<td>Very distinct.</td>
</tr>
<tr>
<td>0.00001</td>
<td>&quot;</td>
<td>Easily perceptible.</td>
</tr>
<tr>
<td>0.001</td>
<td>0.0001</td>
<td>Very strong reaction.</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.00005</td>
<td>Strong reaction.</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.00001</td>
<td>Slight but positive.</td>
</tr>
</tbody>
</table>

Entirely similar results were obtained with glyoxylic acid prepared by the action of water upon silver dichloracetate. Dichloracetic acid was converted into the neutral sodium salt and then precipitated with an equimolecular proportion of silver nitrate. The precipitated silver salt was filtered off with the pump and washed with ice-cold water. It was then scraped into a flask and boiled with water under a reflux condenser. The salt decomposes with formation of glyoxylic acid and dichloracetic acid, which were separated as before. The products reacted in the usual way with tryptophan, indol, and skatol.

\[ 2\text{CHCl}_2\text{COOAg} + \text{H}_2\text{O} = \text{COOH} + \text{CHCl}_2\text{COOH} + 2\text{AgCl} \]

It is difficult to believe that these uniformly positive results are due to impurities and it is therefore concluded that, contrary to the statement of Rosenheim, pure glyoxylic acid reacts with tryptophan and pure sulphuric acid to give the characteristic color reaction.

The products obtained when glyoxylic acid and formaldehyde and oxidizing substances act upon tryptophan and skatol are similar and possibly identical and it is not inconceivable that they are both due to the action of the same substance. Since glyoxylic acid alone gives the reaction, while formaldehyde needs the presence of oxidizing substances, it may be conjectured that

1 Beckurts and Otto, loc. cit.
2 The results are not influenced by boiling the solutions of calcium glyoxylate for some time or by heating the dry salt for an hour at 150° C.
the formaldehyde is converted into glyoxylic acid. If formaldehyde, like acetaldehyde, were to undergo the aldol condensation under the influence of acids, its conversion by oxidation into glyoxylic acid could be readily accounted for.

\[
\text{CH}_2\text{O} \overset{\text{condensation}}{\longrightarrow} \text{HOCH(OH)}\text{C} \overset{\text{oxidation}}{\longrightarrow} \text{CO-OOH}
\]

With the view of testing this possibility, formaldehyde was oxidized with hydrogen peroxide in the presence of strong sulphuric acid, but the formation of glyoxylic acid could not be satisfactorily demonstrated, although the experiments made do not entirely preclude the possibility of its formation. On the other hand it is possible that the glyoxylic acid may undergo decomposition yielding the same products as are obtained from the formaldehyde. Theoretically at least it may be converted into formaldehyde by loss of carbon dioxide, while on the other hand the action of strong sulphuric acid is known to remove carbonic oxide from many acids containing two carbonyl groupings, so that the production of carbonic oxide and formic acid or simply carbonic oxide and water might be considered.

\[
\text{C-OOH} \overset{\text{condensation}}{\longrightarrow} \text{C-H+CO}_2
\]

But neither formic acid nor carbonic oxide, either alone or in conjunction with formaldehyde, react in a typical manner with tryptophan, so that the experimental evidence is at present insufficient to decide whether the chromogenic substance in the case of the glyoxylic and formaldehyde reactions is the same.

Reference may be made to the difference in the action of formaldehyde and glyoxylic acid upon indol in the presence of sulphuric acid. Glyoxylic acid, as has already been mentioned,

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gives a red coloration, whereas formaldehyde gives a violet-blue color very similar in tint to that obtained with glyoxylic acid and tryptophan but which differs from the latter on account of its permanence on dilution with water. This reaction, which has recently been described by Konto,1 differs in other respects from the formaldehyde reaction with tryptophan. Apparently no oxidizing agent is needed as specimens of formaldehyde and pure sulphuric acid which gave no reaction with tryptophan unless a trace of ferric chloride or some other oxidizing substance were added, reacted directly with indol. A further distinction is found in the fact that the reaction with indol is not abolished by a slight excess of formaldehyde, while the tryptophan reaction is not obtained under these conditions. The sensitiveness of the two reactions is approximately the same. Skatol under similar conditions with formaldehyde and pure sulphuric acid gives an ill-defined brownish color, but if a trace of an oxidizing agent be added to the sulphuric acid, the characteristic reddish-pink coloration is obtained.

The preceding experiments have necessarily in some degree trenched upon the line of investigation followed by Dr. F. G. Hopkins and it is with his kind consent that the foregoing observations are published.

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